Production of β-glucosidase using immobilized *Piromyces* sp. KSX1 and *Orpinomyces* sp. 478P1 in repeat-batch culture

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Two anaerobic fungi, one a monocentric strain (*Piromyces* sp. KSX1) and the other a polycentric strain (*Orpinomyces* sp. 478P1), were immobilized in calcium alginate beads and cultured in sequential batches where spent medium (containing 0.25% cellobiose) was repeatedly drained and replaced. β-glucosidase production with KSX1 was maintained for 45 days over six-repeated batch cultures yielding a maximum level of 107 mIU/mL. For 478P1, β-glucosidase production was maintained for 30 days over four repeated batches yielding a maximum level of 34 mIU/mL. Although repeat-batch cultures of KSX1 produced more β-glucosidase than strain 478P1, the maximum specific β-glucosidase produced from these immobilised cultures was similar. The immobilized polycentric strain proved to be operationally superior to strain KSX1, as strain 478P1 did not produce any viable growth in the culture liquor.

Keywords: *Anaerobic fungi; Immobilization; β-glucosidase; Repeat-batch culture; Piromyces; Orpinomyces*
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Introduction

Anaerobic fungi isolated from the digestive tract or faeces of herbivorous mammals, both ruminant and non-ruminant, produce all of the cellulolytic enzyme activities needed for complete cellulose degradation, that is, endoglucanases, exoglucanases and β-glucosidases [11]. The finding that the cellulose solubilization capabilities of *Neocallimastix frontalis* and *Piromyces communis* are better than that of *Trichoderma reesei* [12, 13] provides confidence that the potential for converting cellulose to fermentable sugar may lie in the superior cellulolytic activity of anaerobic fungi.

The most studied method of cellulase production by both aerobic and anaerobic fungi has been batch culture. Batch culture can be coupled with the use of immobilized cells. This approach has been little exploited in cellulase production but appears to hold considerable potential for improving it since immobilization enables the microbial cells to be used repeatedly and continuously [3]. The first report on fungal enzyme production by an immobilized biocatalyst was that of Frein *et al.* [2], who showed that the fungus *Trichoderma reesei* entrapped in κ-carrageenan gel matrix can produce extracellular cellulolytic enzymes. In another study, glucoamylase production by immobilized, pre-grown mycelia of *Aspergillus phoenicus* was shown to be sustained over 5 repeated batches in a total time of 19 days [6].

In a previous investigation we achieved colonization of alginate beads using either monocentric or polycentric types of anaerobic fungi [7]. We found that the two types had different growth habits in calcium alginate gel, providing further insight into the life cycle and growth patterns particular to monocentric and polycentric fungi. This current study is the first to report on the use of these immobilized anaerobic fungi in repeat-batch fermentations and relates these differences in colonization mode to subsequent enzyme productivity.

Materials and methods

Organisms and culture media

The isolation and description of *Piromyces* sp. strain KSX1 and *Orpinomyces* sp. strain 478P1 have been described previously [7]. The cultures were maintained at 39°C in a semi defined ruminal-fluid-free medium [8, 9]. A detailed description of the modifications to this medium (complete medium 10X) and the method for preparation were reported earlier [7].

Cellobiose medium (CM) was used in β-glucosidase production using immobilized cultures and consisted of basal medium 10 (BM; complete medium 10X without carbohydrates) containing 0.25% cellobiose which was separately sterilized at 110°C for 20 minutes in concentrated solutions under a N_2 atmosphere and added aseptically to sterile BM. Glucose medium (GM) was prepared by substituting 0.25% glucose for cellobiose.

Production of immobilized rhizomycelium

Batches of approximately 150 calcium alginate beads (2.5-3.0 mm in diameter) were produced as reported previously [7] using zoospore and partially homogenized rhizomycelial suspensions of strains KSX1 and 478P1, respectively. Sodium alginate (Manugel GMB; Kelco A.I.L., Melbourne, Australia) was prepared as a 3% (w/v) solution dissolved in GM and all washes were performed in BM at 39°C. In immobilised KSX1 cultures alternate repeat-batch and fed-batch techniques using GM were used until fungal biomass was maximized in the

bead. Immobilization of the polycentric fungus was similar to that described for the monocentric isolate but without the need for a culture establishment phase. After 24 hours static incubation at 39ºC, the immobilized 478P1 cultures were shaken on a reciprocating shaker at 80 oscillations per minute.

Repeat-batch production of β-glucosidase using immobilized cells

Repeat-batch fermentation were conducted using duplicate sets of immobilized fungal cultures contained in the same 100 mL serum bottles used in the preparation of immobilized rhizomycelium.

After culture establishment the spent medium in immobilized KSX1 cultures was aseptically removed by sterile anaerobic procedures with 23 gauge needles and 50 mL syringes and the beads were washed with 25 mL BM. Twenty-five millilitres of CM was added and the beads were re-incubated in repeat-batch mode at 39°C statically. Immobilized cultures of strain 478P1 were grown in 25 mL CM as described earlier and subsequently cultured repeatedly in batch mode for enzyme production using the same substrate at the same concentration. All cultures were incubated in a reciprocating water bath at 39°C and shaken.

During repeat-batch fermentation, samples of culture liquor (1.0 mL) were removed by sterile anaerobic procedure at regular the intervals and filtered using 0.2 μm syringe filters (Minisart, Sartorius Australia). The pH of the filtered culture liquor was measured immediately and also assayed for residual cellobiose concentration. β-glucosidase in the culture filtrate was assayed on the day of sampling and when enzyme levels reached a maximum the medium was aseptically removed. After the beads were washed with 25 mL of BM, 25 mL of sterile CM was added to start another batch fermentation with subsequent sampling.

At the end of each batch fermentation duplicate sets of bottles were sacrificed and chitin estimations were performed on them as an indicator of fungal biomass. The beads were washed and the alginate was dissolved in 10% hexametaphosphate (BDH Chemicals Ltd., England). The free fungal rhizomycelium was sedimented by centrifugation at 2000 x *g* for 15 minutes, and the pellets were washed and stored frozen for later chitin analysis.

Free rhizomycelium batch cultures

Free rhizomycelium batch cultures of strains KSX1 and 478P1 were performed in duplicate using 100 mL sealed serum bottles containing 25 mL CM using inocula as for immobilized cultures. Samples of culture liquor (1.0 mL) were taken aseptically at regular intervals for the determination of β-glucosidase and chitin.

Assays

Cellobiose in the culture liquor was determined after enzymatic hydrolysis with β-D-glucosidase glucohydrolase (Sigma) based on the method of Russell and Baldwin [10].

Aryl-β-glucosidase assays were performed using a *p*-nitrophenol derivative of β-D-glucose (5 mM; Sigma) [4]. One β-glucosidase unit (IU) is defined as the amount of β-glucosidase that produced 1 μmole glucose in 1 minute.

For biomass determinations, fungal chitin of immobilized and free cultures was estimated colorimetrically as the total hexosamine content after acid hydrolysis [4].

Microscopy

The surface of the immobilized fungal cultures was observed by scanning electron microscopy at the end of the repeat-batch operation as reported previously [7].

Results

Repeat-batch production of β-glucosidase using immobilized rhizomycelium

β-glucosidase production by immobilized cultures of KSX1 was sustained over six repeat-batch cultures (Figure 1), although free rhizomycelium was present in the culture liquor throughout the duration of the fermentation. In the first batch, β-glucosidase yield was the highest and this levelled off to between 56 and 75% of the initial yield in subsequent batches. Cellobiose was consumed by day 2 in the first batch and day 3 in subsequent batches. Culture pH changed little (6.7 to a minimum of 6.2 within three days).

Immobilized *Orpinomyces* sp. 478P1 maintained β-glucosidase production for 30 days over four successive batches (Figure 1). As with KSX1, enzyme yields declined over the batches (to 59% of the initial yield by the fourth batch). Cellobiose in the medium was consumed slower compared to KSX1 (depleted by day 3 in the first batch then by days 4 to 5 in subsequent batches) and culture pH declined was more pronounced (from 6.7 to 5.9 in four days).

(Insert Fig.1 here)

Figure 1 Production of β-glucosidase by (a) immobilised *Piromyces* sp. KSX1 (!) and (b) *Orpinomyces* sp. 478P1 ( )) grown in repeat-batch cultures.

The amount of immobilized biomass in KSX1 cultures (as indicated by chitin assay) was seven times greater than the amount of free biomass found in the culture liquor (Table 1). The amount of free biomass averaged 0.66mg chitin and remained constant between the repeat-batches. With 478P1, no viable free biomass was found and similar to KSX1, the amount of immobilized biomass in the beads did not vary between batches.

(Insert Table 1)

Table 1 Immobilized biomass, β-glucosidase yields and specific β-glucosidase production in repeat-batch cultures. Each value represents the mean for duplicates.

Specific β-glucosidase production of immobilized rhizomycelium in repeat-batch culture versus unimmobilized rhizomycelium cultures in a single batch culture

Specific enzyme production of immobilized rhizomycelium is given in Table 1 and was calculated from the β-glucosidase yields and chitin values given in this table. These values were compared with specific enzyme production produced at the end of a single batch culture of unimmobilized rhizomycelium for each strain.

Specific β-glucosidase production of unimmobilized single batch KSX1 cultures was 2.0 IU/mg chitin. This was higher than that obtained in the repeat-batch immobilized cultures. Specific β-glucosidase production in the first batch,

calculated from free biomass in the culture liquor was 3.6 IU/mg chitin. This could account for the high β-glucosidase yield in the first repeat-batch of immobilized KSX1 cultures in Figure 1. The specific β-glucosidase production from free biomass in the remaining repeat-batches was approximately equivalent to that found in the single batch unimmobilized culture.

Specific β-glucosidase production of unimmobilized 478P1 cultures was 0.30 IU/mg chitin. In immobilized 478P1 cultures, similar measures of specific β-glucosidase production were obtained at the end of each batch. The exception to this was the increased enzyme activity obtained in the first batch. This is represented as a 42% increase in specific β-glucosidase production using immobilized 478P1 cultures compared to a single batch of free cells. Despite a drop in specific enzyme production in subsequent batches, existing immobilized biomass continued to produce β-glucosidase at levels comparative to free cell cultures.

Bead morphology and containment of rhizomycelia

The gel beads of immobilized KSX1 maintained their shape during the 45-day-long operation. Viable zoospores and rhizomycelium appeared in the culture liquor 24 hours into the incubation of each batch of immobilized KSX1 cultures. Closer examination of the bead surface using scanning electron microscopy (SEM) revealed numerous sporangia associated with an extensive rhizomycelial network (Figure 2a). It is likely that the zoospores were the source of propagules for the development of free rhizomycelia with each addition of fresh media.

Short rhizomycelial segments (ca. 0.5 mm in length) appeared in the culture medium in the second batch of immobilized 478P1 cultures and continued to be produced in subsequent batches. These rhizomycelial segments were found to be non-viable when plated on agar containing M10X medium. A SEM of a gel bead at the end of the fermentation period shows peripheral vegetative rhizomycelial growth on the outside of the bead and no sporangia (Figure 2b).

(Insert Figures 2a and 2b here)

Figure 2 (a) The surface of immobilized *Piromyces* sp. KSX1 after 45 days incubation illustrating numerous sporangia and associated rhizomycelial network. Bar = 20 μm.
(b) The surface of immobilized *Orpinomyces* sp. 478P1 after 30 days incubation. Note the absence of sporangia. Bar = 10μm.

Discussion

This study demonstrates that β-glucosidase can be produced by pre-grown mycelium in repeat-batch culture of the two anaerobic fungi studied. Although the production of β-glucosidase was sustained throughout repeat-batch cultures of KSX1 and 478P1, an increase in enzyme production in the first batch was a phenomenon observed in all repeat-batch experiments and was possibly due to the higher cell viability in the bead that occurred at this time. In examining the physiological aspects of immobilized cells Hahn-Hägerdal [5] has suggested there may be an exponential growth phase with concomitant high metabolic activity immediately after immobilization with only a percentage of the cell population maintaining a high metabolic activity. This population of cells are either in close proximity to the surrounding medium and in a location where space is made available due to the release of cells. Although cell viability may have decreased after the first batch it is likely that peripheral regrowth of both types of anaerobic

fungi on the gel bead may be responsible for maintaining enzyme production during the later batches.

Repeat-batch culture of *Piromyces* sp. KSX1 cells immobilized in alginate gel proved to be a novel method in so far as maintaining production of β-glucosidase over an extended period of incubation. However, the merits of repeat-batch culture of the monocentric fungal culture were limited by the confounding problem of rhizomycelial growth in the culture liquor throughout the fermentation. Although repeat-batch cultures of KSX1 produced more β-glucosidase than strain 478P1, the maximum specific β-glucosidase produced from these immobilised cultures was similar. Free mycelia in KSX1 repeat-batch cultures was contributing to enzyme production, making strain 478P1 a better candidate for immobilization since it did not give rise to viable free mycelium.

Long-term viability has been demonstrated for both strains and is another one of the various advantages encountered when working with immobilized cells. Moreover, the repeat-batch system with immobilized rhizomycelia was operationally more efficient than a single batch culture with free rhizomycelia because immobilization removed the need of re-growing the cells every single batch. In this initial study β-glucosidase production in KSX1 and 478P1 cultures were followed for up to 45 and 30 days respectively. It is possible that the fermentation could have proceeded longer if the incubation was continued considering that the immobilized cultures were still viable.

The alginate beads of both strains of fungi maintained their shape through the repeat-batch cultures. However, it was observed that beads of KSX1 had lost some strength. Immobilized cultures of 478P1 were comparatively stronger due to their ability to invade the whole alginate matrix. The long-term stability of calcium alginate is a factor limiting the wide application of this gel in immobilization and may be associated with the release of free mycelial aggregations in immobilized KSX1 cultures. In a study investigating immobilized *Penicillium chrysogenum* strains in penicillin production [1], it was found that maximum instability of the alginate beads occurred when a decrease of mycelia in the central layers took place by autolysis and the outer layers reached maximal mycelial capacity. This led to a destruction of the beads and the release of the mycelia into the fermentation medium to form a thick suspension.

This study was performed to investigate the use of immobilization of fungal rhizomycelia and repeat-batch cultures as a novel and potentially advantageous approach to cellulase production from anaerobic fungi and has yielded new knowledge on differences between *Piromyces* and *Orpinomyces* in terms of immobilization. Immobilized polycentric fungi may hold particular promise in scale-up operations due to their operational superiority over the monocentric fungi. Future experiments will be required to further define the optimal conditions for extracellular enzyme production by immobilized polycentric anaerobic fungi.

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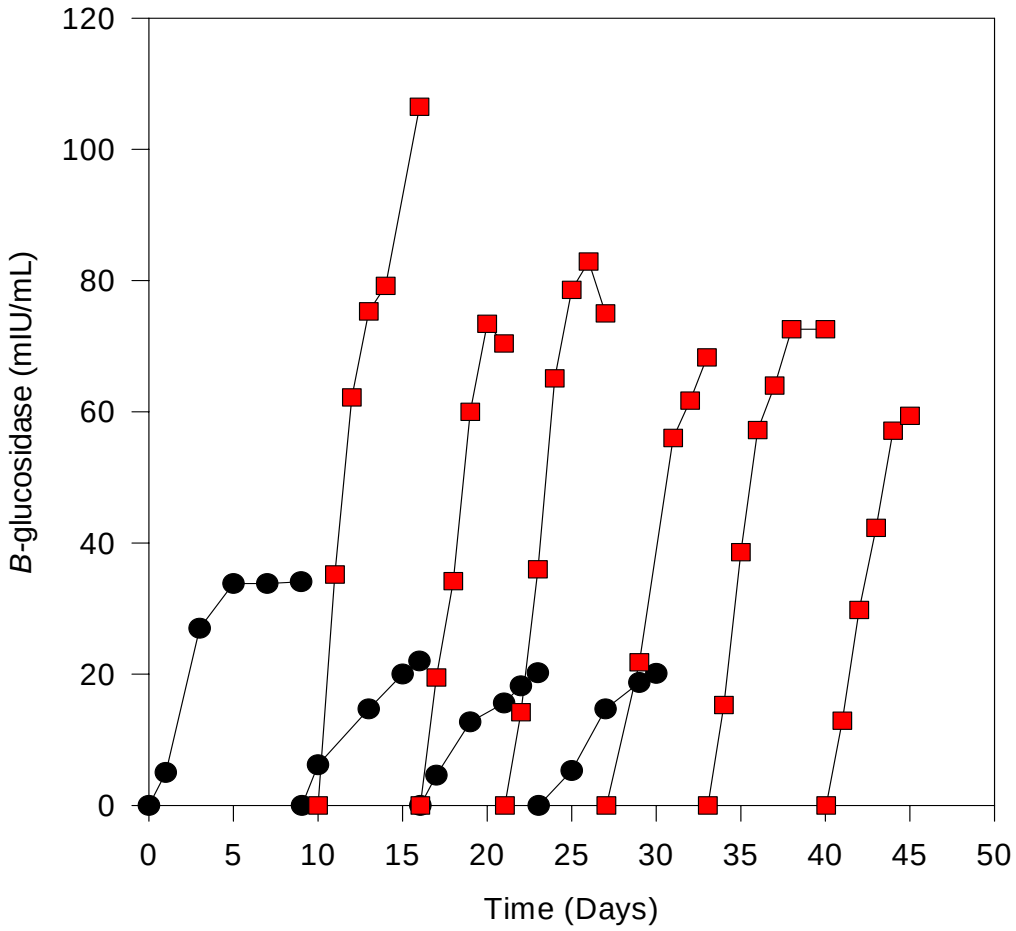
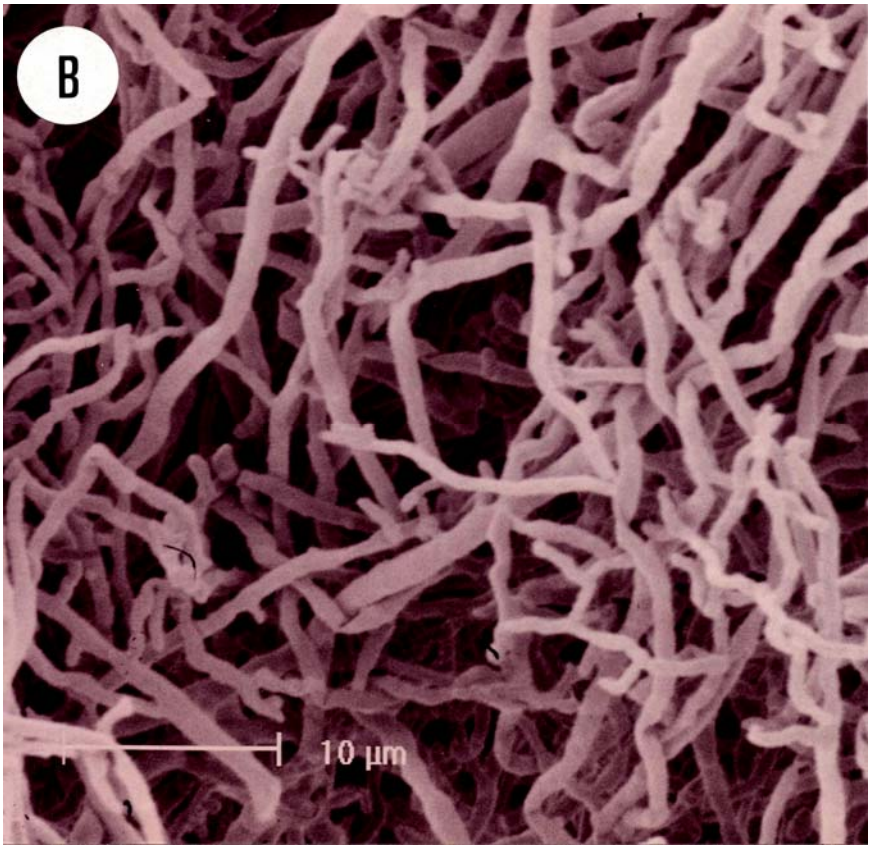


Figure 1 Production of β-glucosidase by (a) immobilised *Piromyces* sp. KSX1 (!) and (b) *Orpinomyces* sp. 478P1 ( )) grown in repeat-batch cultures.

M^{c}Cabe *et al.*, Figure 1

Figure 2
(a) The surface of immobilized *Piromyces* sp. KSX1 after 45 days incubation illustrating numerous sporangia and associated rhizomycelial network. Bar = 20 µm.



M^{c}Cabe *et al*., Figure 2b

(b) The surface of immobilized *Orpinomyces* sp. 478P1 after 30 days incubation. Note the absence of sporangia. Bar = 10µm.

Table 1 Immobilized biomass, β-glucosidase yields and specific β-glucosidase production in repeat-batch cultures. Each value represents the mean for duplicates.

Batch Number[1]	Immobilized Biomass (mg chitin)		β-glucosidase production (mIU/mL)		Specific β-glucosidase production (IU/mg chitin)	
	KSX1	478P1	KSX1	478P1	KSX1	478P1
1	3.90 (0.60)[2]	1.32	106.5	34.1	0.50	0.52
2	4.58 (0.67)	1.30	70.4	22.0	0.31	0.36
3	4.21 (0.73)	1.35	75.0	20.2	0.33	0.3
4	4.52 (0.68)	1.36	68.3	20.1	0.31	0.31
5	4.60 (0.61)		72.6		0.3	
6	4.75 (0.69)		59.4		0.25	

[1] Batch time length as indicated in Figure 1
[2] Free biomass in culture liquor (mg chitin)